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Assistant Commissioner for Patents
Washington, D.C. 20231

On 27 Feb. 2004

TOWNSEND and TOWNSEND and CREW LLP

By: Malinda Adajet

PATENT
Attorney Docket No.: 015280-315100US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

S. Chandrasekharappa *et al.*

Application No.: 09/380,337

Filed: March 6, 2000

For: MENI, THE GENE ASSOCIATED WITH MULTIPLE ENDOCRINE NEOPLASIA TYPE 1, MENIN POLYPEPTIDES AND USES THEREOF

Customer No.: 20350

Examiner: Susan Ungar

Art Unit: 1642

DECLARATION OF DR. SETTARA C. CHANDRASEKHARAPPA UNDER 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Dr. Settara C. Chandrasekharappa being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. I received a Ph.D. in Biochemistry in 1983 from the Indian Institute of Science. I additionally received about 7 years of postgraduate training at St. Louis University Medical Center, the University of Illinois at Chicago, and the University of Chicago.

2. I am currently employed at the National Institutes of Health as Director, Genomics Core, Genome Technology Branch, in the National Human Genome Research Institute. I have worked in the field of human genetics for over 13 years and have authored over 80 peer-reviewed publications in genetics and biochemistry. A copy of my curriculum vitae is provided in Exhibit A.

3. I have read and am familiar with the contents of the application. The claims currently at issue are drawn to nucleic acids that encode menin. I understand that the Examiner has rejected the claims as allegedly not enabled. We had previously submitted papers showing that The rejection appears to be based on the Examiner's belief that there is no connection between the claimed nucleic acid sequences and menin protein. This Declaration provides additional evidence of the association between the nucleic acid and protein sequences.

4. The nucleic acids set forth in SEQ ID NOs:1 and 3 in the specification encode the menin protein of SEQ ID NO:2. We have expressed the protein and obtained antibodies to the protein, which is described in the specification in Example 2. We have also used these reagents (and other menin-specific polyclonal antibodies generated to peptides comprised by SEQ ID NO:2) to evaluate protein expression in various cells and tissues. Exemplary data is presented in Guru *et al., Proc. Natl. Acad. Sci USA* 95:1630-1634, 1998, which is provided in Exhibit B. This study evaluates menin expression in both transfected and untransfected human embryonic kidney cells. As explained on page 1631, column 2 in the first full paragraph, the antibodies detect endogenous protein, which is localized predominantly to the nuclear fraction with a small amount in the membrane fraction. The studies provides additional evidence that the *MEN1* nucleic acids encode menin protein.

5. Additional studies (*e.g.*, Watout *et al, Int. J. Cancer* 85:877-881, 2000) have also shown that menin protein is expressed. Watout *et al.* describe menin polypeptide and nucleic acid sequences with reference to Chandrasekharappa *et al., Science* 276:404-407, 1997 (which is provided as exhibit C) and Guru *et al., supra.* The nucleic acid and polypeptide sequences in the two references are the same as those in the instant application. Thus, one of skill in the art

understands that the protein expression performed by Watout *et al.* relates to the claimed sequences.

6. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true.

Dated: Feb 26, 2004

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PUBLICATIONS

Original Peer-reviewed Papers:

1. **Chandrasekharappa, S.C.**, Smith, J.H. and Eliceiri, G.L. (1983): Biosynthesis of small nuclear RNA's in human cells. J. Cell. Physiol. 117:169-174.
2. **Chandrasekharappa, S.C.**, Gopalakrishnan, A.S., and Jacob, T.M. (1986): Sequence specific antibodies to a deoxyribodinucleotide. Biochem. International 12:173-180.
3. **Chandrasekharappa, S.C.**, and Jacob, T.M.(1986): Purification of antibodies specific to a dinucleotide using the hapten bound to DEAE cellulose as an affinity column. Immun. Investigations 15:1-9.
4. **Chandrasekharappa, S.C.**, Gopalakrishnan, A.S. and Jacob, T.M. (1986): Antibodies specific to deoxythymidine 5'-phosphate. Indian J. Biochem. Biophys., 23:233 -237.
5. Deretic, V., **Chandrasekharappa, S.C.**, Gill, J.F., Chatterjee, D.K. and Chakrabarthy, A. (1987): Vectors for rapid subcloning and expression of Pseudomonas genes. Gene 57:61-72.
6. **Chandrasekharappa, S.C.**, and Subramanian, K.N. (1987): Effects of position and orientation of the 72-bp repeat transcriptional enhancer on replication from the SV40 core origin. J. Virol. 61:2973-2980.
7. Paxton, W.B., **Chandrasekharappa, S.C.**, and Subramanian K.N. (1988): Alteration in the chromatin structure of the origin-promoter region of simian virus 40 concomitant with the shift from early to late gene expression. Mol. Genet. (Life Sci. Adv.) 7:153-158.
8. **Chandrasekharappa, S.C.**, Rebelsky, M.S., Firak, T.A., LeBeau, M.M. and Westbrook, C.A. (1990): A long range restriction map of the interleukin 4 and interleukin 5 linkage group on chromosome 5. Genomics 6:94-99.
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10. Stock, W., **Chandrasekharappa, S.C.**, Neuman, W.L., LeBeau, M.M., Brownstein, B.H., and Westbrook, C.A. (1992): Characterization of yeast artificial chromosomes containing interleukin genes on human chromosome 5. Cytogenet. Cell Genet. 61:263-265.
11. Mercer, J.F.B., Livingston, J., Hall, B., Paynter, J.A., Begy, C., **Chandrasekharappa, S.C.**, Lockhart, P., Grimes, A., Bhave, M., Siemieniak, D., and Glover, T.W. (1993): Isolation of a partial candidate gene for Menkes disease by positional cloning. Nature Genet. 3: 20-25.
12. Yu, H., Thun, R., **Chandrasekharappa, S.C.**, Trent, J.M., Zhang J. and Meisler, M.M. (1993): Human encoding Phosphoenolpyruvate Carboxylase is located on chromosome 20q13.2. Genomics 15: 219-221.
13. **Chandrasekharappa, S.C.**, Gross, L.A., King, S.E., and Collins, F.S., (1993): Localization of the human NME2 gene lies within 18 kb of NME1 in chromosome 17. Genes Chrom. Cancer 6: 245-248.
14. Flejter W., Kukowska-Latallo, J.F., Kiousis, S., **Chandrasekharappa S.C.**, King, S.E. and Chamberlain, J.S. (1993): Tetranucleotide repeat polymorphism at D17S846 maps within 40 kb of GAS at 17q12-22. Human Mol. Genet. 2:1080.
15. Abel K., Boehnke M., Prahalad M, Ho P., Flejter W., Watkins M., VanderStoep J., **Chandrasekharappa S.**, Collins F., Glover T., and Weber B. (1993): A Radiation Hybrid Map of the BRCA1 Region of Chromosome 17q12-21. Genomics 17:632-641.

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17. Flejter, W.L., Watkins, M., Abel, K.J., **Chandrasekharappa, S.C.**, Weber, B.L., Collins, F.S., Glover, T.W. (1993): Isolation and Characterization of Somatic Cell Hybrids with Breakpoints Spanning 17q21.3-q24 by FISH and PCR. Cytogenet. Cell Genet. 64:222-223.
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19. **Chandrasekharappa, S.C.**, King , S.E., Freidman, M.L., Hayes, S.T., Bowcock, A.M. and Collins, F.S. (1993): The CA repeat marker D17S791 is located within 40kb of the WNT3 gene on chromosome17q. Genomics 18:728-729
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21. **Chandrasekharappa, S.C.**, Friedman,L., King, S.E., Lee, Y-H., Welsch, P., Bowcock, A.M., Weber, B.L., King, M-C., Collins, F.S. (1994): The gene for pancreatic polypetide (PPY) and the anonymous marker, D17S78, are within 40 kb of each other on chromosome 17q21. Genomics 21:458-460.
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Cell Biology

Menin, the product of the *MEN1* gene, is a nuclear protein

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ABSTRACT **The *MEN1* gene, mutations in which are responsible for multiple endocrine neoplasia type 1 (MEN1), encodes a 610-amino acid protein, denoted menin. The amino acid sequence of this putative tumor suppressor offers no clue to the function or subcellular location of the protein. We report herein, based on immunofluorescence, Western blotting of subcellular fractions, and epitope tagging with enhanced green fluorescent protein, that menin is located primarily in the nucleus. Enhanced green fluorescent protein-tagged menin deletion constructs identify at least two independent nuclear localization signals (NLS), both located in the C-terminal fourth of the protein. Among the 68 known independent disease-associated mutations, none of the 22 missense and 3 in-frame deletions affect either of the putative NLS sequences. However, if expressed, none of the truncated menin proteins resulting from the 43 known frameshift/nonsense mutations would retain both the NLSs. The precise role(s) of menin in the nucleus remain to be understood.**

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder in which affected individuals variably develop tumors in the parathyroids, anterior pituitary, and enteropancreatic endocrine tissue (1). Recently, we identified the gene responsible for MEN1 (2), and germ-line mutations in this gene have been described for nearly all the fifty-nine MEN1 probands reported so far (3, 4). Also, somatic mutations in the *MEN1* gene have been identified in variable fractions of sporadic parathyroid adenomas, gastrinomas, insulinomas, lung carcinoids, and pituitary tumors (5–8). The nature of the mutations, which are consistent with a loss-of-function mechanism, the observation that the wild-type allele is consistently lost in tumors arising in patients with MEN1, and the observation that both alleles of the *MEN1* gene are often inactivated in sporadic tumors indicate that tumorigenesis is very likely due to loss of function of the *MEN1*-encoded protein menin. Thus the *MEN1* gene seems to be an excellent example of a classic tumor suppressor.

Analysis of the predicted menin amino acid sequence does not show homology to any known protein in the database, nor does it disclose any apparent sequence motifs, providing no clues as to the function of the protein. As a first step toward elucidation of the role of menin in tumorigenesis, we have designed experiments to identify its subcellular location and demonstrate herein that the majority of the protein resides in the nucleus. At least two independent nuclear localization signals (NLSs), both located in the C-terminal fourth of the protein, have been identified by deletion analysis.

MATERIALS AND METHODS

Generation of pcDNA3.1-Menin and EGFP-Menin Constructs. The isolation of the pCMV-Sport menin clone (A11) containing a full-length menin cDNA from a human leukocyte cDNA library has been described (2, 9). The coding region of menin from the A11 clone was amplified by PCR and cloned into the *Eco*RI site of pcDNA3.1(−) Myc-His (Invitrogen) in-frame with and upstream of myc-His epitope sequences to generate pcDNA3.1-menin construct. The same fragment was cloned into the enhanced green fluorescent protein (EGFP) expression vectors (CLONTECH), pEGFP-C2 (see Fig. 3*A*, EGFP1) or pEGFP-N2 to generate menin constructs tagged with EGFP at the N or C terminus, respectively. Each construct was characterized by restriction analysis and sequencing of the coding regions.

Deletion constructs EGFP2–EGFP9 (see Fig. 3*A*) were generated by taking advantage of conveniently placed restriction sites in the construct EGFP1. Constructs EGFP2–EGFP5 were generated by digestion of the plasmid EGFP1 with enzymes *Acc*I, *Apa*I, *Kpn*I, and *Sma*I, respectively, to release fragments representing C-terminal menin deletions of various lengths, followed by recircularization of the remaining larger fragment. Similarly, the N-terminal deletion constructs EGFP7 and EGFP9, as well as the internal deletion construct EGFP8, were generated by using the enzymes, *Bgl*II, *Xho*I, and *Nae*I, respectively. EGFP6 was obtained as a result of a three-way ligation including two *Xho*I fragments, together representing the N-terminal 571 amino acids, to the vector EGFP-C2. The remaining three constructs, EGFP10–EGFP12, were generated starting from EGFP1 by an oligonucleotide-based site-directed mutagenesis method. EGFP10 and EGFP11 were mutants that contain engineered sequences to terminate menin at amino acids 587 and 603, respectively. EGFP12 is engineered to result in the deletion of 12 nucleotides, eliminating amino acids 588–591. The following forward and reverse primers were used to generate constructs EGFP10–EGFP12 by using the Quick Change site-directed mutagenesis kit (Stratagene): EGFP10F, GTCGCAAGTGCAGATGTAGAAGCAGAAAGTGTCC; EGFP10R, GGACACTTTCTGCTTCTACATCTGCACTTGCGAC; EGFP11F, GCACAGTCGCAAGTGCAGATGGTGTCCACCCCTAGTGAC; EGFP11R, GTCACTAGGGGTGGACACCATCTGCACTTGCGACTGTGC; EGFP12F, GACTACACTCTGTCTTTCCTCTAGCGGCAGCGCA; EGFP12R, GCGCTGCCGCTAGAGGAAAGACAGAGTGTAGTC.

Antibodies. Four polyclonal antibodies, KC27, SQV, AEA, and LEE, were generated in rabbits by immunization with synthetic peptides corresponding to menin residues 585–610 (VQMKKQKVSTPSDYTLSFLKRQRKGL), 583–610 (SQVQMKKQKVSTPSDYTLSFLKRQRKGL), 465–492 (AEAEEPWGEEAREGRRRGPRRESKPEEP), and 286–307 (LEELEPTPGRPDPLTLYHKGIA), respectively. Peptide conjugation, immunization, and antibody affinity purification

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Abbreviations: EGFP, enhanced green fluorescent protein; NLS, nuclear localization signal; DAPI, 4,6-diamidino-2-phenylindole.

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on peptide-linked beads were as described (10). Mouse monoclonal antibodies specific for nuclear pore complexes (Babco, Richmond, CA) and tubulin (Oncogene Research Products) were used on immunoblots as markers for nuclear and cytoplasmic fractions, respectively. A monoclonal antibody for the myc epitope was obtained from Invitrogen.

Detection of Menin in Transiently Transfected Cells by Immunofluorescence and Green Fluorescence. Cells (HEK-293T, NIH 3T3, or CHO) were grown on coverslips in DMEM containing 10% fetal calf serum in a 60-mm plate to approximately 40% confluence. Transfection was carried out by using Lipofectamine (Life Technologies) with plasmid DNA (8–10 μg) expressing either full-length or truncated menin, as well as DNA from the respective vectors alone, pcDNA3.1 and EGFP-C2, for immunofluorescence and green fluorescence, respectively. Twenty-four hours after the transfection, cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Fluorescence was visualized by fluorescent microscopy, and the images were collected from a charge-coupled device camera.

For immunofluorescence, cells transfected with pcDNA 3.1-menin on the coverslip as above were washed three times with PBS, blocked with 5% BSA for 5 min, and washed again with PBS, followed by incubation for 2 hr at room temperature with affinity-purified menin antibody (KC27; 20 μg/ml) or myc antibody (1 μg/ml) and 5% BSA in PBS. After washing three times with PBS, coverslips were flooded with fluorescein isothiocyanate-tagged anti-rabbit/anti-mouse secondary antibodies (10 μg/ml) in 5% BSA and incubated for 30 min. After several washes with PBS, cells were stained with DAPI.

Subcellular Fractionation, SDS/PAGE, and Western Blot Analysis of Untransfected and Transiently Transfected Cells. The pCMV-Sport menin clone (A11) containing a full-length menin cDNA isolated from a human leukocyte library (2, 9) was used for transfection. To create a vector-only control, the menin insert was removed by digestion with *Mlu*I and the vector was religated. Flasks (162 cm^2) were seeded with 20–30 million HEK-293T cells per flask in DMEM supplemented with 10% serum 1 day before transfection. Transfection was performed with 10 μg of plasmid DNA (vector with or without menin cDNA) per flask and the Superfect reagent from Qiagen (Santa Clarita, CA). The day after transfection, medium was replaced with DMEM without serum. Forty-eight hours after transfection, cells were harvested by washing with PBS and mechanical scraping from the flask. Cells were pelleted and kept on ice until fractionation. Fractionation of vector-only and menin-transfected HEK-293T cells into nuclear, membrane, and cytoplasm fractions was performed as described (11) with the following modifications: *N*-ethyl maleimide was eliminated from all buffers; aprotinin was set at 0.02%; 1 mM [4-(2-aminoethyl)-benzene sulfonylfluoride] (ICN) was substituted for 1 mM phenylmethylsulfonyl fluoride; and 2-mercaptoethanol was set at 5% (vol/vol). Immunoblot analysis of cell fractions on nitrocellulose membranes was performed after electrophoresis on 10% denaturing polyacrylamide gels (10), using the SQV (2 μg/ml), AEA (5 μg/ml), LEE (5 μg/ml), nuclear pore protein (1 μg/ml), and tubulin (5 μg/ml) antibodies. Protein concentrations of antibodies and cell fractions were determined by a dye binding assay (Bio-Rad).

RESULTS

Detection of Menin in the Nucleus of Cells Transiently Expressing Menin by Immunofluorescence. The complete coding region of menin was cloned into the mammalian expression vector pcDNA3.1, in-frame with the Myc-His epitope sequences, which were placed at the C terminus. HEK-293T (simian virus 40 tumor antigen-transformed human embryonic kidney cells) cells, transiently transfected with the pcDNA 3.1-menin construct, were analyzed by immunofluorescence with antibodies (KC27) raised against a peptide representing the C-terminal 26 amino acids of menin, as well as monoclonal antibodies for the myc epitope. Both antibodies detected fluorescence only in the nucleus (Fig. 1). Similar observations were also made with CHO (Chinese hamster ovary) cells transfected with the same plasmid (data not shown). These results show that menin is located primarily in the nucleus. The menin antibodies failed to detect endogenous menin in untransfected HEK-293T cells by immunofluorescence, presumably due to lower abundance of the protein in individual cells.

Detection of Menin by Western Blotting of Subcellular Fractions. The nuclear localization of menin was confirmed by subcellular fractionation and Western blot analysis. A similar antibody (SQV) raised against a synthetic peptide corresponding to the C-terminal 28 amino acids of menin reacted on immunoblots with full-length menin expressed in *Escherichia coli* (data not shown). SQV antibody was able to detect endogenous menin as an ~67-kDa band localized predominantly to the nuclear fraction with a smaller amount in the membrane fraction of HEK-293T cells (Fig. 2 *Top*). In addition to menin, SQV detects a more rapidly migrating band in the membrane and cytoplasmic fractions but not the nuclear fraction of vector-only transfected cells (Fig. 2 *Top*). We presume this band represents a protein cross-reacting with SQV because its appearance is blocked by the SQV peptide (data not shown), but the protein is not derived from menin itself because its abundance does not increase with menin transfection. In menin-transfected cells, a substantial increase in overall immunoreactivity compared with vector-only transfected cells was observed with SQV antibody. Menin was again predominantly localized in the nuclear fraction, but immunoreactivity was also detected in membrane and cytoplasmic fractions of menin-transfected cells (Fig. 2). In addition to the ~67-kDa menin band, bands of higher and lower mobility were seen with SQV in the nuclear fraction of menin-transfected cells. These appear to be menin-related because their appearance is dependent upon menin transfection. Their significance is uncertain, but the more rapidly migrating band could represent a proteolytic fragment of menin. Western blots with additional antibodies, AEA and LEE, raised against distinct peptides (residues 465–492 and 286–307, respectively) from the menin amino acid sequence confirmed the subcellular distribution of menin in vector-only and menin-transfected cells seen with SQV (data not shown). Because the Western blotting is sufficiently sensitive to detect endogenous menin in the nucleus as well, the nuclear localization observed by immunofluorescence in pcDNA3.1-menin transfected cells is not likely to be an artifact of overexpression.

EGFP-Tagged Menin Deletion Constructs Identify Two NLSs in Menin. EGFP-tagged constructs expressing full-length and truncated versions of menin were constructed. The extent of the menin coding sequence represented and the location of the green fluorescence observed in the transfected cells for each deletion construct is shown in Fig. 3. Stability of the protein product was confirmed by the presence of strong fluorescence in transfected, but not sham-transfected, cells for each construct. HEK-293T cells, transfected with constructs expressing full-length menin tagged with EGFP at either the N terminus (EGFP1) (Fig. 3) or the C terminus (data not shown) were found to generate green fluorescence only in the nucleus. All the C-terminal deletion constructs expressing various lengths of the menin coding region up to amino acid 476 (EGFP2–EGFP5) were found to display green fluorescence in the cytoplasm, indicating that no NLS is present in the first 476 amino acids. However, a construct extending up to amino acid 571 (EGFP6) targets the protein to the nucleus, suggesting that a NLS must be present in the additional 95 amino acids (NLS-1). Analysis of this sequence identified a stretch of 19 amino acids (from positions 479 to 497, RRRG-

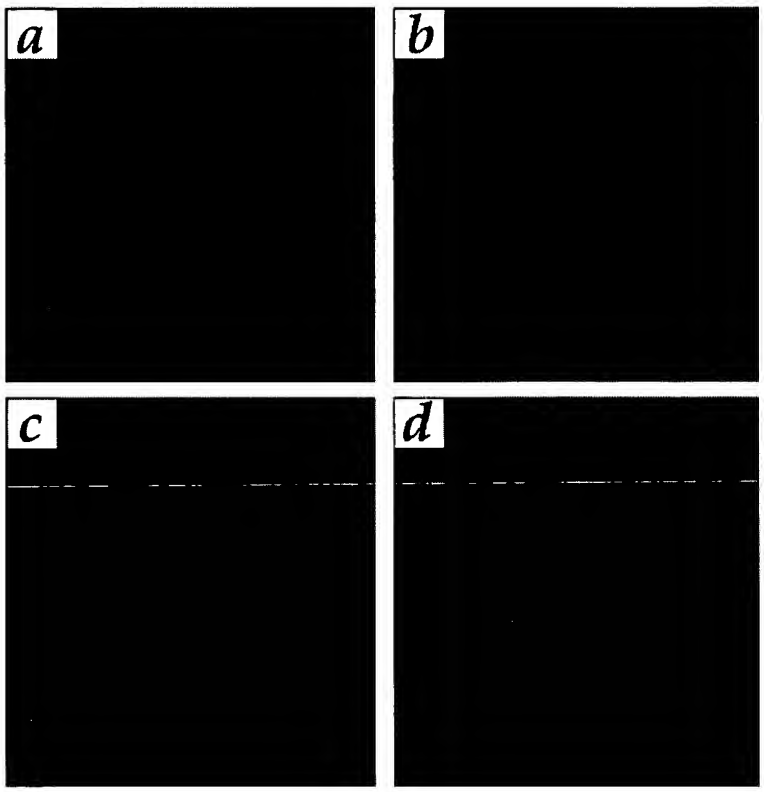

FIG. 1. Immunofluorescence of menin-transfected HEK-293T cells with menin and myc epitope antibodies. Twenty-four hours after transfection with pcDNA3.1-menin, cells were processed for immunofluorescence with menin antibody (KC27) or with myc antibody followed by fluorescein isothiocyanate-conjugated secondary antibody detection. Immunofluorescence pattern with menin antibody (*a*) and the DAPI staining (*b*) showing the nuclei from the same cells. Immunofluorescence with myc antibody (*c*) and the DAPI staining (*d*) showing the nuclei from the same cells. Note that not all cells are positive, because this is a transient transfection. Endogenous levels of menin are not detectable above background.

PRRESKPEEPPPPKK). Four basic amino acid residues out of a stretch of 6 residues can sometimes function as a single basic type NLS signal (12–15), and the region between positions 479 and 485 (RRRGPRR) would fit this. Alternatively, two strings of 2 or 3 basic residues (Arg or Lys) separated by a stretch of 10–12 amino acids have been shown to function in some proteins as a bipartite basic type NLS (13, 15). It is possible that the sequence from positions 484 to 497 (*RR*ESKPEEPPPP*KK*, where the basic residues mentioned are italic) within this stretch of 19 amino acids could function in this manner.

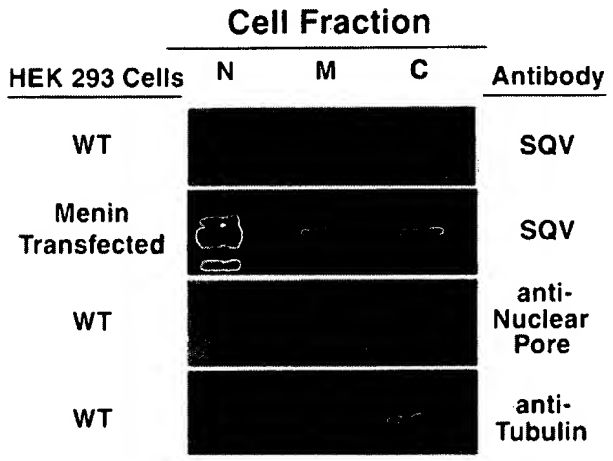

FIG. 2. Immunoblot of representative nuclear (N), membrane (M), and cytoplasmic (C) fractions of HEK-293T cells transfected with vector only (WT) or with menin. Fifty micrograms of protein was loaded for each fraction for the menin blot in WT cells, 5 μg of protein was used for the menin blot in menin-transfected cells, 25 μg of protein is present in each lane for the nuclear pore protein blot, and 12.5 μg of protein was used per lane for the tubulin blot. The blots shown for tubulin and for nuclear pore protein were from WT cells, but blots of fractions from menin-transfected cells gave comparable results.

Analysis of N-terminal deletion constructs identified another NLS (NLS-2) within the C-terminal 39 amino acids of menin, as can be seen from the results of construct EGFP9 (Fig. 3). The sequence from positions 588 to 608 (*KKQK*VSTPSDYTLSFL*KRQRK*) in this region could provide a bipartite NLS and/or the sequence from positions 604 to 608 (KRQRK) could function as a single basic type NLS. Three additional constructs that eliminate NLS-2 completely (EGFP10) or delete either component of this bipartite signal (EGFP11 and EGFP12) still targeted menin to the nucleus, presumably because they all still carried NLS-1.

DISCUSSION

Immunofluorescence, Western blot analysis of subcellular fractions, and epitope tagging with EGFP indicate that menin is primarily located in the nucleus. Many proteins that localize to the nucleus contain a polybasic motif, the NLS, which is necessary for proper nuclear targeting (12–15). Although no precise match to the consensus NLS sequence is present in menin, we hypothesized that such signal(s) might exist. Studies with EGFP-tagged menin deletion constructs indicate that at least two functionally independent NLSs exist in the menin protein. There are several prior examples of proteins carrying multiple functional NLSs. For instance, SRY and SOX9, members of the family of high-mobility group domain transcription factors, each contain two independent NLSs (16).

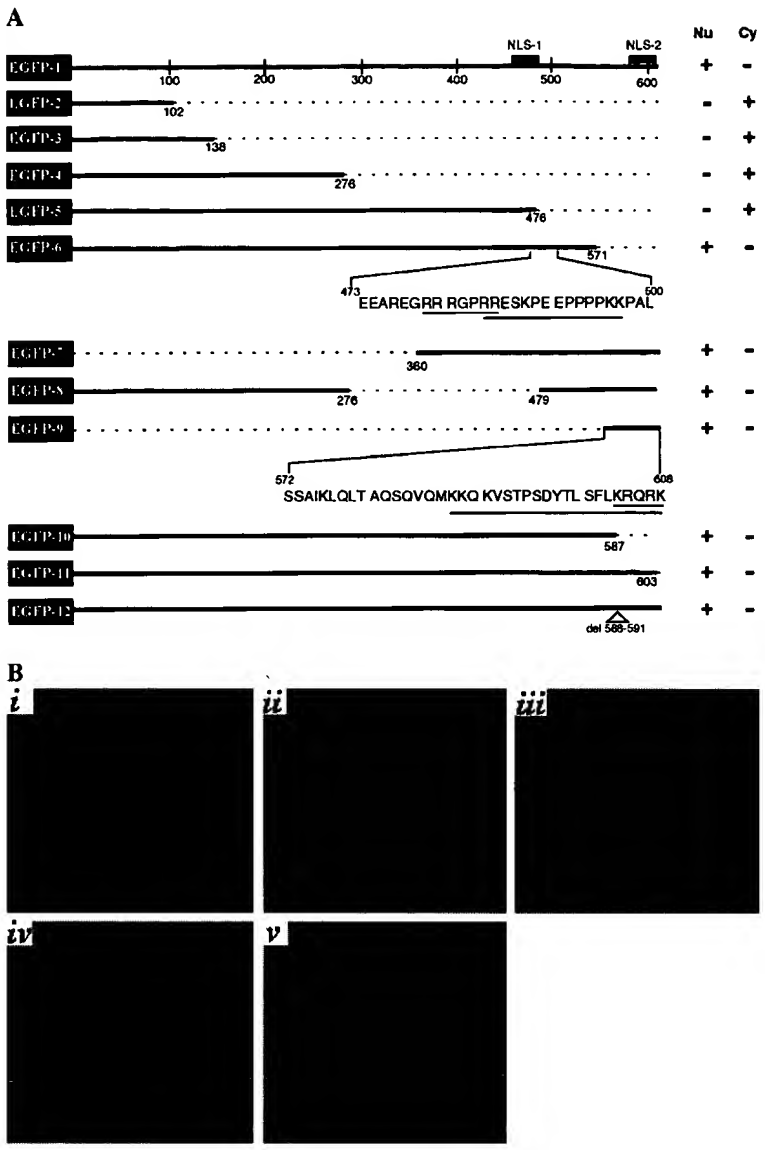

FIG. 3. (*A*) Schematic of deletion constructs of menin fused to EGFP and localization of green fluorescence observed in the transfected cells. All constructs (EGFP1–EGFP12) were generated with EGFP (boxed) at the N terminus of the menin coding region. The extent of the menin coding region retained in each construct is represented by a solid thick line and the deleted region is represented by a dotted thin line. The position of the starting and the terminating amino acid in each deletion construct is also indicated, except for amino acids 1 and 610. The presence (+) or absence (−) of green fluorescence observed in the nucleus (Nu) or cytoplasm (Cy) for each construct is shown. The sequence around the two putative independent NLSs mapped in this study (NLS-1 and NLS-2) are shown for constructs EGFP6 and EGFP9, respectively. Underlined sequences represent potential single basic type and bipartite sequences in both NLS-1 and NLS-2. (*B*) Representative examples of green fluorescence in HEK-293T cells transfected with EGFP-menin fusion constructs. The presence of green fluorescence in the nucleus or cytoplasm of cells transfected with EGFP1 (*i*), EGFP5 (*ii*), EGFP6 (*iii*), and EGFP9 (*iv*) is shown. The diffuse pattern of fluorescence observed in cells transfected with EGFP vector alone (*v*) is also shown

Both NLS-1 (amino acids 479–497) and NLS-2 (amino acids 588–608) are present in the C-terminal fourth of menin. However, none of the known 22 missense and 3 in-frame germ-line or somatic MEN1 single amino acid deletion mutations fall within either of the putative NLS-1 and NLS-2 sequences (3–8). Thirty-nine of the 43 known frameshift/nonsense mutations would result in a truncated menin lacking both NLS-1 and NLS-2 and, therefore, should be retained in the cytoplasm. The truncated proteins from the four most distal mutations should retain the NLS-1 and, if expressed, would be predicted to localize to the nucleus. Because there are no discernible phenotypic variations associated with these four MEN1 mutations (3, 5, 7), it is unclear whether there is any functional significance to whether a truncated menin protein is located in the nucleus or cytoplasm.

It is possible that some truncated mutant proteins present in MEN1 patients are unstable and degraded. However, the clear signal either in the nucleus or cytoplasm from the cells expected to express truncated EGFP-tagged proteins suggest that truncated versions of menin are partially stable, at least in this cell culture system.

The nuclear localization of menin suggests a variety of possible functions, such as serving as a component in transcriptional regulation, DNA replication, or cell cycle control. Certainly there are many other tumor suppressor genes whose normal protein products are found in the nucleus. But the precise role played by menin in the regulation of endocrine cell growth control will require additional investigation.

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38. Order Primates Linnaeus 1758; suborder Anthropoidea Mivart 1864; infraorder Catarrhini Geoffroy, 1812; superfamily Hominoidea Gray 1825; family not assigned. Genus *Morotopithecus* gen. nov. **Generic diagnosis:** Large hominoid, male weight around 40 to 50 kg, most comparable in facial and dental morphology to *Proconsul* and *Afropithecus*. Differs from later Miocene and extant apes in having a longer midface and has less alveolar prognathism than extant large apes. Differs from *Afropithecus*, later Miocene hominoids, and extant apes in the greater degree of cingular development on cheek teeth, especially molars. Differs from *Afropithecus* and *Proconsul* in having a narrower interorbital region and larger premolars relative to M1; differs from *Proconsul* in a smaller M2 and M3 relative to M1. Differs from *Afropithecus* in possessing a shorter premaxilla, a higher face, a broader nasal aperture (*5*), a P^3 that is much broader buccally and in which the paracone is situated closer to the protocone, buccal wrinkling on the side of the molars, and a larger M^3. Judging from worn occusal surfaces, the enamel was intermediate thin as in *P. major* (*51*). Further, computed tomography scans on the skull of *Afropithecus* reveal a thick palate with a small incisive canal (*52*), an anatomical condition that is distinctly different from the large canal of the Moroto palate (*53*). The glenoid is rounder in shape and more like that of extant apes and atelines than can be inferred for Miocene hominoids other than *Oreopithecus* and possibly *Dryopithecus (42)*. Lumbar morphology differs from that of *Proconsul* and resembles that of *Oreopithecus*, possibly *Dryopithecus*, and extant large apes and siamangs in transverse process position. Proximal femoral morphology resembles that of cercopithecines and primitive hominoids such as *Proconsul*, differing from that of extant apes. Distal femoral anatomy resembles that of *Proconsul*, *Kenyapithecus*, and extant apes in mediolateral breadth but differs from hylobatids, *Proconsul*, and *Kenyapithecus* in the buttressing of the intercondylar notch. In 1962, L. S. B. Leakey referred the Moroto palate to *Pseudogorilla* (*54*) but did so without any species diagnosis. *Pseudogorilla* was created by Elliot in 1912 for ape specimens from the "Upper Congo" (*55*), now referred to *Gorilla*. Regardless of the validity of *Pseudogorilla*, the Moroto fossil is clearly different from any extant ape. **Type species:** *Morotopithecus bishopi* sp. nov. **Etymology:** Moroto, after Moroto township in Karamoja District in Uganda, and *pithekos* from the Greek for ape; and after the late W. W. Bishop. **Type specimen:** UMP 62-11 (UMP, Ugandan Museum of Paleontology), a palatofacial specimen with all teeth. **Type locality:** Moroto II. **Distribution:** Early Miocene, Karamoja District, NE Uganda. **Hypodigm:** Type and UMP 62-10 and UMP 66-01, which are probably associated mandibular fragments; UMP 62-12, left upper canine; UMP 67-28, middle lumbar vertebra; UMP 68-05, middle lumbar vertebral body; UMP 68-06, a last thoracic vertebra; UMP 68-07, the lamina and base of a spine of a lumbar vertebra; MUZM 80, right and left femoral pieces (all from the Moroto II locality); and MUZM 60, scapular fragment with glenoid (Moroto I locality). **Specific diagnosis:** As for genus.
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56. We thank the many helpful individuals in Uganda, including the Office of the President, the National Research Council, and the staff at the Zoology Museum at Makerere University, as well as C. Chapman for the many efforts made on our behalf. We thank D. Aleper for assistance in the field; B. Masek for assistance in the laboratory; M. Mehrer and J. Flynn; and the American School of Prehistoric Research and the L. S. B. Leakey Foundation for contributing funding to this project.

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Positional Cloning of the Gene for Multiple Endocrine Neoplasia–Type 1

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Multiple endocrine neoplasia–type 1 (MEN1) is an autosomal dominant familial cancer syndrome characterized by tumors in parathyroids, enteropancreatic endocrine tissues, and the anterior pituitary. DNA sequencing from a previously identified minimal interval on chromosome 11q13 identified several candidate genes, one of which contained 12 different frameshift, nonsense, missense, and in-frame deletion mutations in 14 probands from 15 families. The *MEN1* gene contains 10 exons and encodes a ubiquitously expressed 2.8-kilobase transcript. The predicted 610–amino acid protein product, termed menin, exhibits no apparent similarities to any previously known proteins. The identification of *MEN1* will enable improved understanding of the mechanism of endocrine tumorigenesis and should facilitate early diagnosis.

Familial cancer syndromes have attracted widespread interest over the past decade, in part because of their potential to shed light on the general mechanisms of carcinogenesis. Positional cloning methods have led to the precise identification of the responsible gene for more than a dozen such disorders (*1*). In keeping with the hypothesis originally articulated by Knudson for retinoblastoma (*2*), most of the responsible genes are of the tumor suppressor type. In such a circumstance, affected individuals have inherited one altered copy of the responsible gene from an affected parent, but the tumors have lost the remaining copy (the wild-type allele) as a somatic event. Thus, the inheritance pattern is dominant, but the mechanism of tumorigenesis is recessive. The importance of gene discovery often extends beyond affected pedigrees, as the same tumor suppressor gene is often found to play a role (by mutation of both alleles) in sporadic cases of the same neoplasm.

Multiple endocrine neoplasia–type 1 (MEN1) (OMIM *131100) appears to be a compelling example of this paradigm, with prevalence estimates ranging from 1 in 10,000 to 1 in 100,000 (*3*, *4*). Affected individuals develop varying combinations of tumors of parathyroids, pancreatic islets, duodenal endocrine cells, and the anterior pituitary, with 94% penetrance by age 50 (*4*). Less commonly associated tumors include foregut carcinoids, lipomas, angiofibromas, thyroid adenomas, adrenocortical adenomas, angiomyolipomas, and spinal cord ependymomas. Except for gastrinomas, most of the tumors are nonmetastasizing,

but many can create striking clinical effects because of the secretion of endocrine substances such as gastrin, insulin, parathyroid hormone, prolactin, growth hormone, glucagon, or adrenocorticotropic hormone.

Nine years ago *MEN1* was mapped (*5*) to chromosome 11q13 by linkage analysis (Fig. 1A). Subsequent investigation of a large number of pedigrees by many groups revealed no evidence of locus heterogeneity (*6*, *7*). The identification of critical recombinants recently led to the conclusion that the candidate interval is bounded by marker D11S1883 on the centromeric side and marker D11S449 on the telomeric side (*7*) (Fig. 1B).

In a concerted effort to identify *MEN1*, we developed 18 new polymorphic markers in the *MEN1* region of 11q13 (*8*) and constructed a fully overlapping 2.8-Mb contig map of yeast, bacteriae, and P1 artificial chromosome (YAC, BAC, and PAC) clones and P1 clones (*9*). We then carried out an intensive search for transcripts, which resulted in the identification of 33 candidate genes (*10*). To focus the search more precisely, we also took advantage of the observation that tumors arising in MEN1 patients are frequently found to have somatically lost the wild-type allele of markers in the vicinity of the gene (*5*, *11*). Interstitial deletions or mitotic crossing-over events of this sort provide information on candidate interval boundaries. We used tissue microdissection to separate tumor cells from stroma (*12*) in a large number of familial MEN1 tumors and sporadic gastrinomas, and we found an entirely consistent minimal interval (Fig. 1B) bounded centromerically by marker PYGM (*12–14*) and telomerically by marker D11S4936 (*14*).

We analyzed the sequence of two BACs (b137C7 and b79G17) covering most of this interval (Fig. 1C) (*15*), as well as publicly available sequence of a few cosmids just telomeric to b79G17 (*16*). A total of eight transcripts were identified by comparison with expressed sequence tag (EST) databases and computer analysis for the likely presence of exons. Each of these transcripts was considered a possible candidate for *MEN1*.

One of these eight candidates, originally designated *mu*, was first identified by PowerBLAST matches (*17*) between shotgun sequence assemblies derived from b137C7 and 44 different ESTs in the dbEST database. Twenty-six of these ESTs were human clones isolated from seven different tissues; the remaining 18 ESTs were derived from mouse or rat libraries. Interestingly, 20 of the human ESTs had previously been as-

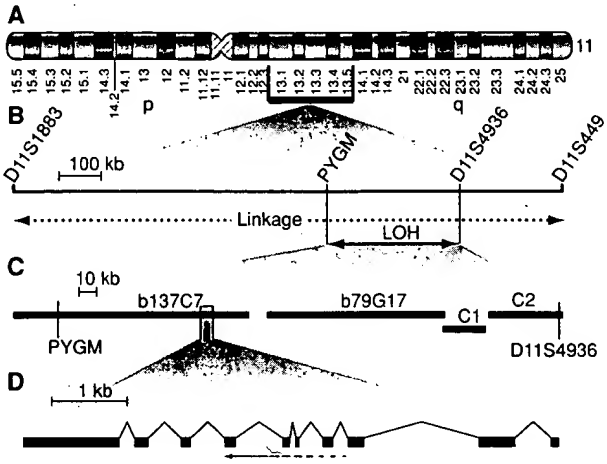

Fig. 1. Steps in the positional cloning of the *MEN1* gene. Initial linkage to chromosome 11q13 (**A**) led to finer mapping by meiotic recombination and tumor loss of heterozygosity (LOH) analysis (**B**). Nearly complete bacterial clone coverage of the most likely candidate interval (PYGM to D11S4936) was achieved with BACs b137C7 and b79G17 and cosmids cSRL116b6, 23c9, and 114g4 (*16*), which could be assembled into two sequence contigs, C1 and C2 (**C**). DNA sequencing revealed several candidate genes, one of which (**D**) was found to harbor mutations in 14 of 15 probands. The arrow indicates the direction of transcription.

Fig. 2. Predicted amino acid sequence of the protein encoded by the *MEN1* gene, as derived from an apparently full-length leukocyte cDNA clone. The first methionine is associated with an excellent Kozak (*26*) consensus sequence (GCCATGG), and no other in-frame ATG codons are found upstream. The GenBank accession numbers for the cDNA (2772 bp) and genomic (9181 bp) sequences are U93236 and U93237, respectively.

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MGLKAAQKTL FPLRSIDDVV RLFAAELGRE EPDLVLLSLV LGFVEHFLAV NRVIPTNVPE   60
LTFQPSPAPD PPGGLTYFPV ADLSIIAALY ARFTAQIRGA VDLSLYPREG GVSSRELVKK  120
VSDVIWNSLS RSYFKDRAHI QSLFSFITGT KLDSSGVAFA VVGACQALGL RDVHLALSED  180
HAWVVFGPNG EQTAEVTWHG KGNEDRRGQT VNAGVAERSW LYLKGSYMRC DRKMEVAFMV  240
CAINPSIDLH TDSLELLQLQ QKLLWLLYDL GHLERYPMAL GNLADLEELE PTPGRPDPLT  300
LYHKGIASAK TYYRDEHIYP YMYLAGYHCR NRNVREALQA WADTATVIQD YNYCREDEEI  360
YKEFFEVAND VIPNLLKEAA SLLEAGEERP GEQSQGTQSQ GSALQDPECF AHLLRFYDGI  420
CKWEEGSPTP VLHVGWATFL VQSLGRFEGQ VRQKVRIVSR EAEAAEAEEP WGEEAREGRR  480
RGPRRESKPE EPPPPKKPAL DKGLGTGQGA VSGPPRKPPG TVAGTARGPE GGSTAQVPAP  540
AASPPPEGPV LTFQSEKMKG MKELLVATKI NSSAIKLQLT AQSQVQMKKQ KVSTPSDYTL  600
SFLKRQRKGL                                                         610
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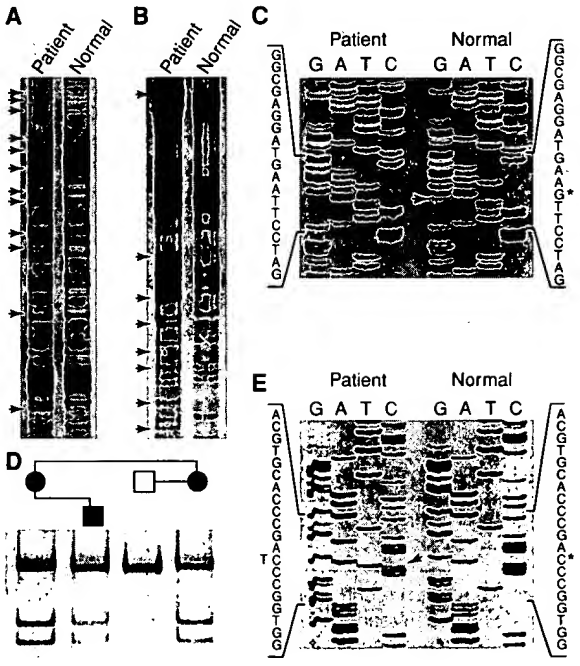

Fig. 3. Detection of frameshift and nonsense mutations. (**A**) Analysis of exon 2 in a MEN1 patient and a normal control, using ddF to reveal pattern differences (arrows) indicative of a possible mutation (*20*). (**B**) Abnormal ddF pattern in exon 9 from a different patient. (**C**) Identification of a single nucleotide deletion by sequencing of a cloned exon 2 PCR product from the patient whose ddF pattern is shown in (A). The sequence shown is of the antisense strand; the mutation is 512delC. (**D**) This frameshift mutation was confirmed by detecting the presence of a new AfI II site in PCR-amplified exon 2 from this patient and two affected relatives. (**E**) Direct sequencing of the exon 9 PCR product from (B), revealing the presence of a heterozygous C → T substitution. Again the sequence is of the antisense strand; the mutation creates a stop codon (TGG → TAG or W436X).

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sembled into a UniGene cluster and placed on the transcript map between markers D11S913 and D11S1314 (*18*).

These 26 human ESTs constituted a 1.9-kb cDNA contig. Northern (RNA) blotting (*10*) identified a transcript of 2.8 kb that was expressed in roughly equivalent amounts in all adult tissues tested, including pancreas, adrenal medulla, thyroid, adrenal cortex, testis, thymus, small intestine, stomach, spleen, prostate, ovary, colon, and leukocytes. Screening of a leukocyte cDNA library yielded an apparently full-length 2.8-kb clone whose sequence was then fully determined on both strands (Fig. 2). Comparison of the cDNA sequence with genomic sequence from b137C7 revealed that the *mu* gene contains 10 exons (with the first exon untranslated) and extends across 9 kb (Fig. 1D).

Primers designed from intronic sequence were used to amplify exons from genomic DNA of affected members of 15 typical MEN1 families (*19*), and mutations were sought by the dideoxy fingerprinting (ddF) method (*20*). Two examples of abnormal ddF patterns are shown in Fig. 3, A and B (exons 2 and 9). Sequencing of polymerase chain reaction (PCR)–amplified material (Fig. 3E), or in some instances cloned products (Fig. 3C), was used to identify the nature of the abnormality. For 10 different mutations for which other affected family members were available for study (all except E363del and W436X), we confirmed that the observed alteration was inherited concordantly with the MEN1 phenotype (Fig. 3D) (*21*).

A total of five frameshift mutations, three nonsense mutations, two in-frame deletions, and two missense alterations were identified (Fig. 4). Two mutations (416delC and 512delC) were encountered twice in families not known to be related. None of these mutations were observed in an analysis of 71 normal DNA samples. Four relatively common polymorphisms—R171Q (CGG/CAG), L432L (CTG/CTA), D418D (GAC/GAT), and A541T (GCA/ACA)—were also encountered and were observed in 1.4%, 0.7%, 42%, and 4% of normal chromosomes, respectively (n = 142).

The identification of mutations in 14 of 15 unrelated affected individuals leaves little doubt that the *MEN1* gene has been identified. We propose the name menin for the 610–amino acid predicted protein product. Sequence analysis provides few clues to its normal function. There is no signal peptide, and, although there are four moderately hydrophobic regions in the NH_2-terminal half of the protein, these are not likely to represent transmembrane domains. Three leucine-rich regions match the PROSITE signature for leucine zippers (*22*), but these regions are not amphipathic and have no strong coiled-coil potential, and this signature is known to generate many false positive matches. Nuclear localization signatures are absent. The protein sequence has several regions of low compositional complexity, including a very hydrophilic mixed-charge cluster between residues 446 and 491 (*23*). There is no detectable homology to the complete genomic sequence of *Saccharomyces cerevisiae*.

The observation that many of the mutations detected (Fig. 4) would most likely result in loss of function of the protein product is consistent with a tumor suppressor mechanism. Such a mechanism distinguishes MEN1 from the related disorder multiple endocrine neoplasia–type 2, where activating mutations of the *RET* oncogene are responsible (*24*). Although, in the absence of examples of complete gene deletion, we cannot rule out the possibility of a dominant negative effect of the truncated menin protein product, the observation of mutations in which as few as 82 amino acids would be left intact (357del4, Fig. 4) makes this mechanism unlikely. It will be of great interest to determine whether, as predicted by the Knudson model (*2*), somatic mutations in the *MEN1* gene are responsible for sporadic endocrine tumors, including the common parathyroid adenomas, which occur at an annual incidence of 154 per 100,000 in individuals over age 60 (*25*).

Now that the *MEN1* gene has been cloned, it will be important to study the role of *MEN1* gene diagnostics in younger at-risk individuals so as to assess the value of identifying or excluding the presence of a mutation before the onset of symptoms. Moreover, the application of a broad and powerful repertory of molecular genetic, cell biological, and animal model approaches can now be initiated to pursue an understanding of the molecular basis of this disorder, with the eventual goal of developing better therapeutic strategies.

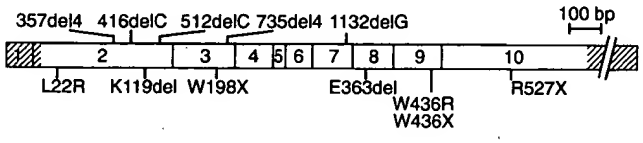

Fig. 4. Summary of mutations identified in 15 unrelated MEN1 patients. The locations of the five frameshift mutations are shown above a diagram of the *MEN1* gene, with the exons numbered; cross-hatched areas are untranslated. Two in-frame deletions of a single amino acid, three nonsense mutations, and two missense mutations are shown below the gene diagram. The 416delC and 512delC mutations were each encountered twice. Mutation abbreviations follow standard nomenclature (*27*).

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15. BAC clones were from the Research Genetics library (Huntsville, AL). BAC DNA was purified by means of a cleared-lysate diatomaceous earth method [H. Q. Pan *et al.*, *Genet. Anal. Tech. Appl.* **11**, 181 (1994)]. Sequencing was undertaken using the double-stranded, shotgun-based approach [A. Bodenteich, S. Chissoe, Y. F. Wang, B. A. Roe, in *Automated DNA Sequencing and Analysis Techniques*, M. D. Adams, C. Fields, J. C. Venter, Eds. (Academic Press, London, 1994), pp. 42–50]. The resulting sequences were screened to eliminate vector, assembled into contiguous fragments, and proofread using the Phred/Phrap/Consed system developed by P. Green (http://chimera.biotech.washington.edu/uwgc/). Contigs larger than 1 kb were deposited before publication in the "unfinished" division of the high-throughput genome sequencing (HTGS) GenBank database with no restriction on public access. Accession numbers are AC000134 and AC000159 for BACs b13767 and b79G17, respectively. Completion of the BAC sequences is still in progress.
16. These cosmid sequences are available at http://mcdermott.swmed.edu.
17. All assembled contigs larger than 1000 bases were analyzed with the program PowerBLAST (J. Zhang and T. Madden, *Genome Res.*, in press; ftp://ncbi.nlm.nih.gov/pub/sim2/PowerBlast/), which masks low-complexity sequences and repetitive elements and then performs simultaneous BLASTN and BLASTX searches, reporting the results in graphical form. Both the nr and est databases at http://ncbi.nlm.nih.gov/were searched. Contigs were also analyzed with the program GRAIL (http://avalon.epm.ornl.gov/) and the programs FEXH and HEXON (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html).
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19. The diagnosis of MEN1 was based on the presence of tumors in two of the three principal systems (parathyroid, enteropancreatic endocrine tissue, or anterior pituitary). Diagnosis of familial MEN1 required at least one first-degree relative with a tumor of one or more of these systems. There were 1 to 47 living affected members in each kindred, with a median of 5. All participating family members gave full informed consent in a protocol approved by the NIDDK Institutional Review Board.
20. Genomic DNA was isolated from blood samples with the Qiagen Kit (Chatsworth, CA). Exons 2 through 10

were amplified individually or in groups from genomic DNA by means of primers designed from intron sequences (supplementary PCR primer and ddF primer sequences can be found at www.sciencemag.org or www.nhgri.nih.gov). PCR was performed in 25-μl reactions containing 100 ng of DNA and 0.5 U of AmpliTaq Gold (Perkin-Elmer) according to the manufacturer's protocol. Dimethyl sulfoxide (final concentration 5%) was included for exons 2, 9, and 10. The primary PCR products were subjected to a dideoxy chain termination reaction with 200 μM dideoxyguanosine triphosphate (Boehringer Mannheim) and AmpliTaq Gold, as described [G. H. Sarkar, H. J. Yoon, S. S. Sommer, *Genomics* **13**, 441 (1992)], with modified gel running conditions. The ddF reactions were diluted 1:4 in buffer containing 7 M urea, 50% formamide, bromophenol blue, and xylene cyanol. Reactions were heated at 94°C for 5 min and chilled on ice, and a 5-μl sample was loaded on a nondenaturing gel [0.75× Mutation Detection Enhancement (MDE) (FMC Bioproducts, Rockland, ME) in 0.5× tris-borate EDTA (TBE)] on a sequencing apparatus. The gel was electrophoresed at a constant power of 8 W at room temperature in a buffer system (consisting of 0.5× TBE in the top reservoir and 0.8× TBE with 0.5 M sodium acetate in the bottom reservoir) until the bromophenol blue reached the bottom of the gel. The gel was removed on Whatman paper, dried for 30 min in a sequencing gel drier, and autoradiographed overnight. One ddF primer could screen about 250 bp; if the region to be screened in the primary PCR product was larger, additional primers were used for ddF. Samples showing changes in band patterns were subjected to cycle sequencing with the same primary PCR product and the same end-labeled primer as was used in the ddF reaction. For insertion or deletion type changes in which the actual bases involved could not be ascertained from the sequence of the heterozygous patient sample, the primary PCR product was cloned in the TA cloning vector pCRII (Invitrogen) and then sequenced.

21. Confirmation that the mutation segregated with MEN1 was achieved by direct sequencing of PCR products from other affected family members. Independent confirmation of the sequence change in affected individuals was achieved by restriction digestion of the appropriate exon PCR product for 512delC (creates an Afl II site), W436R (creates Msp I and Nci I sites), and R527X (creates a Bsu 36I site). For the remainder, analysis was carried out with radioactively labeled allele-specific 16- to 20-nucleotide oligomers, corresponding to the wild-type or mutant sequence, that were hybridized to slot blots of exon PCR products as described [J. Lyons *et al.*, *Science* **249**, 655 (1990)].
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28. This paper is dedicated to the memory of Gerald D. Aurbach. We thank all the MEN1 families who participated and the clinicians (NIDDK–National Institute of Child Health and Human Development NIH Interinstitute Endocrine Training Program, NCI Surgery Branch, and Clinical Center Diagnostic Radiology Department) who helped care for them. We thank C. Cummings, N. Dietrich, L. Gieser, B. Pike, C. Robbins, and S. Saggar for technical support, S. Sommer for advice on the ddF procedure, D. Leja for assistance in preparing the illustrations, and P. Fakunding for manuscript preparation. Supported by the intramural research programs of NHGRI, NIDDK, NCI, and NLM, the Fritz Thyssen Stiftung Fund (C.H.), and a U.S. Department of Energy Graduate Fellowship (J.S.C.).

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Endosomal Targeting by the Cytoplasmic Tail of Membrane Immunoglobulin

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Membrane-bound immunoglobulin (mIg) of the IgG, IgA, and IgE classes have conserved cytoplasmic tails. To investigate the function of these tails, a B cell line was transfected with truncated or mutated γ2a heavy chains. Transport to the endosomal compartment of antigen bound by the B cell antigen receptor did not occur in the absence of the cytoplasmic tail; and one or two mutations, respectively, in the Tyr-X-X-Met motif of the tail partially or completely interrupted the process. Experiments with chimeric antigen receptors confirmed these findings. Thus, a role for the cytoplasmic tail of mIg heavy chains in endosomal targeting of antigen is revealed.

The B cell antigen receptor (BCR) is a multiprotein complex that includes the membrane-bound immunoglobulin molecule (mIg) and the Ig-α,Ig-β heterodimer (*1*). The latter molecules function as the signaling subunit of the BCR. They are also required for the intracellular transport of IgM-BCR to the endosomal compartment, where the bound antigen is proteolytically degraded (*2*). All classes of mIg are associated with the Ig-α,Ig-β heterodimer (*3*), but the heavy chains differ in the length of their cytoplasmic tails: there are 3 amino acids for μm and δm tails and 28 amino acids for γm and εm tails. No function has so far been attributed to the conserved cytoplasmic sequence of mIgG molecules that are expressed on memory B cells.

To analyze the function of the 28–amino acid cytoplasmic tail of the γ2am heavy chain, we truncated or mutated the sequence (*4*) coding for this tail in the expression vector pSV2neoγ2am (*5*). The chain lacking all cytoplasmic amino acids except for the three KVK (*6*) residues (which are identical to the COOH-terminus of the μm chain) we called γ2amtl. Point mutations were introduced to change the YXXM motif in the γ2am cytoplasmic sequence to either LXXM (γ2amY20L) or LXXL (γ2amY20L,M23L). Expression vectors for these heavy chains were transfected into K46λ12 B lymphoma cells expressing a λ1 light chain. The expressed wild-type and mutated γ2am chains associate with the λ1 light chain to form 5-iodo-4-hydroxy-3-nitrophenyl-acetyl (NIP)–specific mIgG2a molecules.

After surface biotinylation of K46λγ2am and K46λγ2amtl cells, the wild-type and tailless IgG2a-BCR complexes were affinity-purified over NIP-Sepharose (*7*) and analyzed by protein immunoblotting (Fig. 1). This analysis confirmed that the γ2amtl chain has a lower molecular weight than the wild-type γ2am chain (Fig. 1, lanes 4 and 2) and showed that both mIgG2a molecules are associated with the Ig-α,Ig-β heterodimer to the same extent. Yet unidentified surface proteins of 41 and 42 kD were copurified together with the wild-type but not truncated IgG2a-BCR complex. These molecules may thus require the γ2am tail for efficient binding. A fluorescence-activated cell sorter analysis confirmed that similar amounts of mIgG2atl and wild-type mIgG2a were expressed on K46 cells, whereas the two point-mutated mIgG2a molecules were expressed in amounts that were reduced by a factor of 3 to 5.

The endosomal transport of antigen bound to wild-type or mutated IgG2a-BCR was tested in an ovalbumin (OVA) peptide presentation assay (*8*). The different γ2am transfectants of K46λ12 cells were cocultured with the T helper cell line 3DO54.8, which is specific for the OVA 323-339 peptide in the presence of NIP-OVA or OVA alone (Fig. 2). The K46λγ2am cells, which express wild-type IgG2a-BCR, were able to present the antigenic peptide to the T cells when exposed to low amounts of NIP-OVA, whereas exposure to the same amount of OVA did not result in antigen presentation (Fig. 2B; $P < 0.001$). K46λγ2amtl cells, which express the tailless IgG2atl-BCR complex, did not present the OVA peptide even when cultured with large amounts of the specific antigen (Fig. 2C). The same defect was found in two independent γ2am transfectants of K46λ12 that expressed an IgG2a-BCR with a double (Y → L, M → L) (*6*) mutation of the YXXM motif. These are referred to as K46λγ2amY20L,M23L (Fig. 2, E and F). K46λγ2amY20L cells, expressing an IgG2a-BCR with a single Y → L mutation of the YXXM motif, had a modest but not statistically significant capacity to present antigen (Fig. 2D; $P < 0.3$). The

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